REMARKS

Applicants' again wish to thank the Examiner for the courtesy of conducting an interview with applicants' representative on December 23, 2009. As indicated on the interview summary, the examiner and the undersigned discussed the prior art of record and proposed claim amendments including proposed amendments to claim 18.

Claims 18 and 20-35 are in this application. Claims 1-17 and 19 are cancelled and claims 31-34 are withdrawn. Claim 18 has been amended to include that steps a), b) and c) are carried out in sequence and that the DNA denaturing solution and the lysis solution are different. Support for new claim 35 is found on page 14, lines 30-32.

The Examiner considers that claims 18, 20, 21, 27 and 29 are anticipated by Spano and these same claims are anticipated by Januskaukas et al. These rejections are respectfully traversed.

There are two important differences in claim 18 such that claim 18 and the claims dependent thereon are not anticipated by Spano and Januskaukas.

1. The present invention comprises two sequential treatment steps of the sample:

- a denaturing step (using a denaturing solution) followed by
- a lysis step (using a lysis solution).

This is set out in parts a) and b) of claim 18.

Claim 18: A method to evaluate the integrity of chromatin or DNA of sperm cells of an animal comprising the following steps in sequence:

a) <u>treating a sample containing the sperm, with a solution of DNA denaturing solution,</u>

b) <u>a single treatment step of treating the sample in the solution obtained in step a) with a lysis solution</u> to extract nuclear proteins of the sperm cells, <u>wherein the lysis solution does not contain protein denaturing detergents</u>, and

c) evaluating the integrity of the chromatin or DNA of the sperm cells based on measurement of halo size of the sperm cells.

Both Spano et al. and Januskauskas, et al describe only one treatment step (a denaturing step using a denaturing solution).

It is also clear that in this invention, the DNA denaturing solution is not the same as the lysis solution. If the solutions were the same then steps a) and b) of the claimed process could be combined.

2. The present invention comprises an evaluation step based on the measurement of the halo size.

Spano et al. comprises an evaluation step based on the use of a methacromatic staining (AO), and flow cytometry to discriminate between different types of fluorescence.

As explained in the previous response, the method defined in Spano et al. consists of 1) treating the sample with a denaturing detergent solution (0.17% Triton x 100, 0.15 M NaCl, 0.08 N HCl, pH 1,4) (see page 30, col. 2, line 9-12) and 2) staining the sample with AO. The technique used in the method disclosed in Januskaukas et al. is the same as the method described in Spano et al. (see page 951, reference 34 of Januskaukas et al.).

Therefore, prior to the staining step, only one treatment step (a denaturing step with a denaturing solution) is carried out. There is not subsequent lysis step, using a lysis solution, as in the present invention.

It is applicants' opinion that the examiner considers that components of both solutions (denaturing and lysis) are present in the only solution used in the method of Spano and Januskaukas. Thus, the examiner reaches the conclusion that the use of a denaturing solution followed by use of a lysis solution is equivalent to the use of one solution.

However, as explained below this is not correct.

First, it is important to understand the function of each of the solutions used in the method of the present invention.

Denaturing solution has the function of denaturing sperm DNA and separating DNA strands. Based on the level of DNA fragmentation, denaturation will be more or less intense as the single-strand and double strand breaks increase the susceptibility to such a solution. Therefore, DNA with high fragmentation degree will be more denatured than non fragmented DNA.

Protamines are sperm nuclear proteins that attach to sperm DNA in order to pack it. Lysis solution removes protamines and, thus, expands DNA halos. The expansion is different depending on whether DNA is denatured or not, so different size halos are obtained.

In the documents cited by the examiner only a denaturing solution is used, wherein Triton acts as membrane-permeabilizing agent not as a lysis agent. Even if Triton was considered as a lysis agent, applicants would like to clarify that the use of a solution, being a denaturing and a lysing solution at the same time, is not capable of producing the differential effect obtained in the method of the present invention for the following reasons:

- Using only one solution, the denaturant effect does not occur prior to lysis, so that differential denaturation according to the level of DNA fragmentation, as described in the present invention, cannot be produced prior to lysis.
- When the components of the lysis solution exert their function to extract nuclear proteins, DNA cannot have been differentially denatured in terms of their level of fragmentation.

The procedure of the present invention has been carried out using two different solutions in two sequential steps: 1) denaturing solution, and 2) lysis solution. The procedure used is :

1) Include the sperm in an agarose matrix on a pretreated slide.
2) A treatment step of the sperm with a denaturing solution
3) A treatment step of the denatured sperm with a lysis solution, which does not contain protein denaturing detergents, and
4) Staining with Wright dye and to visualize by microscopy.

In figure 2 (attached), cells with halos can be perfectly distinguished from cells without halos.

Therefore, there is no such differential expansion of halos according to their fragmentation. As can be seen in Figure 1 attached hereto, wherein only one solution (lysis + denaturing) has been used, all cells show big halos of similar sizes, independently of the DNA fragmentation.

To put it in colloquial terms, if a single solution (single-step treatment of the sample) is used, the procedure would not allow the denaturing components to have time to "exercise its role" and denature the DNA differentially depending on the fragmentation before lysis occurred. Therefore, removal of protamines and the release of halos would occur without prior differential denaturation of DNA, which would make all the halos essentially equal as shown in Figure 1. That is, a solution such as that disclosed in the cited documents, would produce the same halo in all sperm and would not permit the study of the level of DNA fragmentation in terms of the halo size (as in step c) of claim 18.

The applicants' understand that the Examiner considers that the Spano's solution (0.17% Triton x 100, 0.15 M NaCl, 0.08 N HCl, pH 1,4) is a solution that contains all those components used in applicants' lysis and denaturation steps. Thus, the Examiner considers that the use of only this solution, in a single step, is equivalent to the use of two different solutions in two sequential steps. In order to show that these are not equivalent, a solution with denaturing and lysing components was prepared by mixing them together to prepare a single solution. The procedure used is:

1) Including the sperm in an agarose matrix on a pretreated slide,
2) a treatment step of the sperm, with a denaturing solution and a lysis solution, wherein said lysis solution does not contain protein denaturing detergents, and
3) Staining with Wright dye and to visualize by microscopy

The results can be seen in Figure 1.

Denaturing components cannot perform its role before lysis occurs, and thus, all cells appear with halos.

Therefore, the act of first using a denaturing solution, and then a lysis solution, provides important differences that bring advantages to the method of the invention, such as direct visualization of the size of the halos (See Figure 2 attached), wherein two sequential solutions (first denaturing and then lysis solution) have been used. Here, different size halos can be clearly seen, depending on the DNA fragmentation).

As explained above, the present invention also comprises an evaluation step based on the measurement of the halo size. The examiner insists that an evaluation step based on visual determination of the size of the halo of sperm cells is also included in Spano and Januskaukas.

Applicants do not at all agree with this statement. A person skilled in the art knows that these are two different techniques.

Spano and Januskaukas disclose a method to study the sperm chromatin quality, both before and after swim-up and after cryopreservation. For this end, Spano et al use the sperm chromatin structure assay (SCSA), already described by Evenson and Jost (1994) (see page 30, first line under "flow cytometric analysis" subtitle).

As was explained previously, the SCSA is a flow cytometric (FCM) technique which exploits the methachromatic properties of Acridine Orange (AO) to monitor the susceptibility of sperm chromatin DNA to an *in situ* acid partial denaturation. This method consists on 1) treating the sample with a denaturing detergent solution (0.17% Triton x 100, 0.15 M NaCl, 0.08 N HCl, pH 1,4) (see page 30, col. 2, line 9-12) and 2) staining the sample with AO.

Thus, the treated sample is stained with AO and evaluated by flow cytometry. This AO staining emits a green fluorescence when it binds with double stranded DNA while, in the sperm with denatured DNA, in single strand, this fluorochrome emits a red fluorescence. Therefore, the

sperm with denatured DNA are quantified using flow cytometry, to discriminate between both types of fluorescence (page 30 and 31, under subtitle "Flow cytometric analysis..."). And this is what is detected by the cytometer: intensities at certain wavelengths, that is, red or green sperm heads or with different proportions of red and green. Halos of chromatin or DNA cannot be visualized nor quantified because there are not. Chromatin loops continue as they were, i.e. packed into the head. The sperm head still has the same appearance as always.

The method of the present invention does not use a methacromatic staining, as AO, and flow cytometry is not used to discriminate between different types of fluorescence.

In the present invention, the evaluation step is a visual analysis, based on the measurement of the halo sizes. The essential features of the method of the invention, the use of a two sequential steps a denaturing step and a lysis step allow a better preservation of the morphology of the head, or core, obtaining dispersion halos. Further, the tail is maintained, allowing differentiating sperm cells from other kind of cells. The evaluation can be carried out routinely, by conventional microscopy.

Therefore, the method of the present invention can measure the size of the halos of dispersion through microscopy, while the method of the cited documents measures the fluorescence intensity by a flow cytometer. They are two completely different techniques.

In the background of the present invention, those differences between the method of the present invention and the method of Spano et al. (Evenson et al.) are detailed (page 3, lines 12-35). Further, the advantages derived from the method of the present invention as compared with Evenson et al technique are also disclosed (page 4, lines 1-5).

In Evenson et al, 1999. (Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Human Reproduction vol.14 no.4 pp.1039-1049) (page 1041, 2nd paragraph), SCSA technique is described. Nothing about halos and sizes are detailed:

"The extent of DNA denaturation was quantified by the calculated parameter at [at 5 red/(red 1 green) fluorescence; Darzynkiewicz et al., 1975]. Normal, native chromatin remains structurally sound and produces a narrow at distribution. DNA in spermatozoa with abnormal chromatin structure has increased red fluorescence (Evenson et al., 1980, 1985) which yields an at distribution which is usually broader, having a higher mean channel (Xat) and a larger percentage of cells outside the main population of cells (COMPat). Standard deviation of at (SDat) describes the extent of chromatin structure abnormality within a population. Mean green fluorescence reflects DNA content and/or degree of sperm chromatin condensation, the latter because it excludes DNA stainability."

Thus, the SCSA method and the method of claim 18 do not measure anything remotely similar.

Therefore, as all elements of the claims are not included in the cited references, the references cannot anticipate the claims and it is respectfully requested that the rejections be withdrawn.

It is submitted that the application is in condition for allowance and favorable consideration is respectfully requested.

Respectfully submitted,

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